Abstract

To date, there is no protective vaccine for Ebola virus infection. Safety concerns have prevented the use of live-attenuated vaccines, and forced researchers to examine new vaccine formulations. DNA vaccination is an attractive method for inducing protective immunity to a variety of pathogens, but the low immunogenicity seen in larger animals and humans has hindered its usage. Various approaches have been used to improve the immunogenicity of DNA vaccines, but the most successful, and widespread, is electroporation. Of increasing interest is the use of molecular adjuvants to produce immunomodulatory signals that can both amplify and direct the immune response. When combined, these approaches have the possibility to push DNA vaccination into the forefront of medicine.

Key words *DNA vaccination, delivery methods, electroporation, molecular adjuvants*, *intramuscular, intradermal*

1. Introduction

The *Filoviridae* family of viruses is composed of enveloped RNA viruses with nonsegmented, negative-sense genomes. Filoviruses are divided into three serologically distinct genera: *Ebolavirus, Marburgvirus,* and *Cuevavirus* [1]. The *Ebolavirus* genus is composed of five species including *Zaire ebolavirus* (EBOV), *Sudan ebolavirus* (SUDV), *Tai Forest Virus* (TAFV), *Reston ebolavirus* (REBOV) and *Bundibugyo ebolavirus* (BDBV), all of which cause disease except REBOV. EBOV is considered the most lethal species with a mortality range of 60-90% in human outbreaks [2]. The *Marburgvirus* genus currently includes a single viral species, *Marburg marburgvirus*, which is lethal in 70-85% of cases. It is defined by two viruses, Marburg virus (MARV) and Ravn virus (RAVV). *Cuevavirus* genus has a single viral species, *Lloviu cuevavirus*, and one defined virus, Lloviu virus (LLOV). Due to the high mortality rate, person to person

transmittability, and potential to instill panic within the public, a concerted effort has been made to develop an effective filovirus vaccine.

Early efforts to design a protective EBOV vaccine were not completely successful due to a lack of knowledge surrounding the correlates of protective immunity, but progress has recently been made. The viral envelope glycoprotein, GP, is the main target of antibody responses [1]. Numerous studies have demonstrated that humoral immunity confers protection against filovirus challenge in both rodent and non-human primate (NHP) models [3-8]. Conversely, recent studies utilizing knockout murine models have suggested that cell-mediated immune responses are critical in controlling filovirus infections [9, 10]. Therefore, it is believed that truly protective filovirus vaccines must elicit both arms of the adaptive immune response. The ability of plasmid DNA vaccination to induce both humoral and cell-mediated immune responses has made DNA vaccines an attractive platform for generating protective immunity against filovirus infection [11].

The demonstration of effective DNA vaccination in small animal models changed the paradigm for vaccine technology. For the first time, genes encoding antigens, instead of antigens themselves, were shown to elicit broad immunity against a variety of pathogens. DNA vaccination has many advantages over other established vaccination platforms. Foremost is the endogenous production and presentation of antigen without danger of toxicity or infection, a common concern with live attenuated and sometimes with inactivated viral vaccines. As antigen is produced in vivo, antigen presentation occurs on both class I and class II major histocompatibility complex (MHC) molecules, yielding both $CD4^+$ and $CD8^+$ T cell responses. Therefore, DNA vaccination may yield a balanced, protective immune response that is similar to that of authentic viral infection.

Whereas small animal studies have proven the utility of DNA vaccines delivered by intramuscular injection, similar studies in larger animals and humans have been less successful. This has led to the

investigation of alternative approaches capable of increasing both the strength and duration of vaccine-induced immune responses (*see* **Note 1**). The most promising methods of DNA vaccination are intramuscular (IM) injection or intradermal (ID) injection of DNA, diluted in saline buffer, followed by electroporation. Delivery of an electrical pulse to cells transiently opens pores in the lipid bilayer, allowing DNA to travel down the concentration gradient and cross the cellular membrane. Electroporation increases gene expression up to 1000-fold when compared to animals receiving only IM injection. Furthermore, electroporation has the added benefit of increasing DNA distribution throughout the tissue and initiating a pro-inflammatory reaction at the site of injection, thereby propagating the immune response [12-15].

Another approach that has been effective in increasing DNA vaccine immunogenicity is the use of "vaccine cocktails" containing the DNA vaccine as well as plasmids encoding immunomodulatory proteins. Molecular adjuvant plasmids expressing cytokines, chemokines, or co-stimulatory molecules may be co-administered with the antigenic DNA vaccine plasmid (*see* **Note 2**). Cells transfected by molecular adjuvant plasmids secrete the adjuvant into the surrounding region, stimulating both local antigen presenting cells and cells in the draining lymph node. This results in sustained, low level production of immune modulating cytokines that can tailor the immune response towards a more desirable outcome. A wide range of inflammatory and helper T cell cytokines have been studied in small animal models in conjunction with DNA vaccination [16, 17], however, a few in particular stand out for their potential in human vaccination [18].

Here we present a detailed method for delivering an antigen/adjuvant DNA vaccine cocktail in conjunction with electroporation. Importantly, this protocol may be used with a wide range of antigens, providing a strong foundation for future vaccine research.

2. Materials for DNA Vaccine Delivery

2.1. DNA Vaccine

1. Endotoxin free, calcium and magnesium free phosphate buffered saline
2. DNA plasmid coding for the antigenic transgene dissolved in endotoxin free phosphate buffered saline to appropriate concentration (*see* **Note 3**).
3. DNA plasmid coding for the molecular adjuvant transgene dissolved in endotoxin free phosphate buffered saline to appropriate concentration (*see* **Note 3**).

2.2 Electroporation

1. Appropriate anesthesia or tranquilizer for animal (e.g. ketamine or isoflurane gas).
2. Electric razor to shave skin at site of injection (*see* **Note 4**).
3. U-100 insulin syringe with 29G1/2 needle or tuberculin syringe with 28G needle.
4. Electropulse generator
5. Electrode needle array
    a. Intramuscular injection array
    b. Intradermal injection array

3. Methods for DNA Vaccine Delivery

3.1. Intramuscular electroporation of mice

1. Mix the appropriate amount of antigen encoding DNA plasmid and molecular adjuvant encoding plasmid to obtain the desired ratio of antigen to adjuvant. For bilateral vaccination, you will need a maximum of 100 µL for each mouse (*see* **Note 5**).
2. Adjust the electroporation parameters according to the manufacturer's suggested values (*see* **Note 6**).
3. Draw DNA solution into syringe (*see* **Note 7**).

4. Anesthetize the animal with the appropriate anesthesia or tranquilizer (*see* **Note 8**).
5. Shave the fur over the anterior, lateral, and medial surfaces of the leg.
6. Position the animal on its back or side. Extend the leg to provide increased access to the tibialis muscle. A bent or crooked leg will limit the success of the injection and subsequent electroporation.
7. Insert the needle and syringe into the center of the electrode array (integrated array). Alternatively, some electroporation systems may lack space for the needle and syringe in between the microarray. In this case, the injection must be delivered first, and then the electrode microarray is positioned around the site of injection and the electropulse is delivered. It is useful to mark the site with a dark colored marker.
8. Align the needle/electrode array along the middle of the tibialis muscle. Insert both the needle and electrodes into the muscle. Needle should be inserted between 2-3 millimeters into the muscle.
9. Slowly inject the DNA solution (i.e. between 5-10 seconds) without changing the pressure exerted on the syringe plunger.
10. Administer the electrical pulse. The number of pulses, pulse length, magnitude, and interval are dependent on both the animal and the manufacturer of the electroporation device (*see* **Note 9**).
11. Retain the needle and electrode array in place for a few additional seconds upon completion of injection/pulse delivery to prevent the seepage of vaccine from the site of injection.

3.2. Intramuscular electroporation of nonhuman primates

1. Mix the appropriate amount of antigen encoding DNA plasmid and molecular adjuvant encoding plasmid to obtain the desired ratio of antigen to adjuvant. Usually, between 200 µL and 1 mL is delivered.
2. Adjust the electroporation parameters according to the manufacturer's suggested values (*see* **Note 6**).
3. Draw DNA solution into syringe (*see* **Note 7**).
4. Anesthetize the animal with the appropriate anesthesia or tranquilizer (*see* **Note 8**).
5. Shave the area over the injection site. The deltoid, biceps, and quadriceps are the most common regions and have been shown to successfully result in immune responses.
6. Position the animal on its back or side. Extend the limb to provide increased access to the muscle. A bent or crooked limb will limit the success of the injection and subsequent electroporation.
7. Insert the needle and syringe into the center of the electrode array (integrated array). Alternatively, some electroporation systems may lack space for the needle and syringe in between the microarray. In this case, the injection must be delivered first, and then the electrode microarray is positioned around the site of injection and the electropulse is delivered. It is useful to mark the site with a dark colored marker.
8. Align the needle/electrode array along the middle of the muscle. Insert both the needle and electrodes into the muscle.
9. Slowly inject the DNA solution (i.e. between 5-10 seconds) without changing the pressure exerted on the syringe plunger.
10. Administer the electrical pulse. The number of pulses, pulse length, magnitude, and interval are dependent on both the animal and the manufacturer of the electroporation device (*see* **Note 9**).

11. Retain the needle and electrode array in place for a few additional seconds upon completion of injection/pulse delivery to prevent the seepage of vaccine from the site of injection.

3.3. Intradermal electroporation of mice

1. Mix the appropriate amount of antigen encoding DNA plasmid and molecular adjuvant encoding plasmid to obtain the desired ratio of antigen to adjuvant. The maximum volume recommended for intradermal vaccination of mice is 20-25 µL per mouse (*see* **Note** 5).
2. Adjust the electroporation parameters according to the manufacturer's suggested values (*see* **Note** 6).
3. Draw DNA solution into syringe (*see* **Note** 7).
4. Anesthetize the mouse with the appropriate anesthesia or tranquilizer (*see* **Note 8**).
5. Position the mouse on its stomach and shave the fur over the lower back (*see* **Note 10**).
6. Insert the needle and syringe into the center of the electrode array (integrated array). Alternatively, some electroporation systems may lack space for the needle and syringe in between the microarray. In this case, the injection must be delivered first, and then the electrode microarray is positioned around the bleb and the electropulse is delivered.
7. Stretch the skin taut between the thumb and index finger.
8. Align the needle/electrode array along the middle of the shaved patch. Insert both the needle and electrodes into the epidermal region of the skin. Needle should be inserted between 2-3 millimeters into the skin. The needle should be inserted bevel up, parallel to the skin, and then rotated 90^{o}. This results in a shallow delivery of drug to the epidermal layer of the skin. To assist in positioning, a pair of forceps may be used to pinch the skin.

9. Slowly inject the DNA solution (i.e. between 5-10 seconds) without changing the pressure exerted on the syringe plunger (*see* **Note 11**).
10. Administer the electrical pulse. The number of pulses, pulse length, magnitude, and interval are dependent on both the animal and the manufacturer of the electroporation device (*see* **Note 9**).
11. Retain the needle and electrode array in place for a few additional seconds upon completion of injection/pulse delivery to prevent the seepage of vaccine from the site of injection.

3.3. Intradermal injection of nonhuman primates

1. Mix the appropriate amount of antigen encoding DNA plasmid and molecular adjuvant encoding plasmid to obtain the desired ratio of antigen to adjuvant. The maximum volume for intradermal injection of nonhuman primates is 100 µL.
2. Adjust the electroporation parameters according to the manufacturer's suggested values (*see* **Note** 6).
3. Draw DNA solution into syringe (*see* **Note** 7).
4. Anesthetize the animal with the appropriate anesthesia or tranquilizer (*see* **Note 8**).
5. Shave the area over the injection site. For nonhuman primates, the skin covering the back and limbs is considered the optimal site of injection.
6. Insert the needle and syringe into the center of the electrode array (integrated array). Alternatively, some electroporation systems may lack space for the needle and syringe in between the microarray. In this case, the injection must be delivered first, and then the electrode microarray is positioned around the bleb and the electropulse is delivered.
7. Stretch the skin taut between the thumb and index finger.

8. Align the needle/electrode array along the middle of the shaved patch. Insert both the needle and electrodes into the epidermal region of the skin. Needle should be inserted between 2-3 millimeters into the skin. The needle should be inserted bevel up, parallel to the skin, and then rotated 90^{o}. This results in a shallow delivery of drug to the epidermal layer of the skin. To assist in positioning, a pair of forceps may be used to pinch the skin.
9. Slowly inject the DNA solution (i.e. between 5-10 seconds) without changing the pressure exerted on the syringe plunger (*see* **Note 11**).
10. Administer the electrical pulse. The number of pulses, pulse length, magnitude, and interval are dependent on both the animal and the manufacturer of the electroporation device (*see* **Note 9**).
11. Retain the needle and electrode array in place for a few additional seconds upon completion of injection/pulse delivery to prevent the seepage of vaccine from the site of injection.

Notes

1. There are several methods for increasing DNA plasmid protein production. Inclusion of Kozak sequences upstream of the start site, or removal of any ATG codon sites in the 5' untranslated region may increase the rate of transcription. Likewise, codon optimization has proven extremely successful in increasing the rate of protein synthesis. Conversely, commercial expression vectors are available. These do not generally require much optimization, although depending on the antigen and model used, they may not be the ideal choice. Careful thought should be given to developing the vaccine plasmid, and it may be necessary to perform preliminary studies to confirm the overall expression and immunogenicity of the DNA vaccine.
2. Incorporation of molecular adjuvants (i.e. cytokines) is not always straight forward. It is important to remember that cytokines are species specific and must often times be designed to account for this. For cloning purposes, PCR primers should be designed to recognize the most conserved sequences across multiple species.
3. Phosphate buffered saline is preferable to water, as water can relax the super-coiled state of the DNA plasmid.
4. Shaving of the fur is essential for proper visualization of the injection site. The best electric razors for mice are those with closely spaced teeth. Regular veterinary or dog grooming razors are suitable for larger animals as they do not get tangled in long hair as easily.
5. Allow for dead space within the syringe by preparing enough DNA for n+3 doses. Insulin syringes are preferable because they have little dead space. Tuberculin syringes have about 100 µL of dead space and often develop air bubbles, making it difficult to accurately deliver the appropriate amount of DNA. A volume of 50 µL/site is the most optimal, as this allows for proper distribution of the vaccine plasmid within the muscle without causing damage to the tissue itself. Additionally, larger volumes may cause the DNA vaccine to leak out of the muscle, limiting the immune response.

6. It may be necessary to perform preliminary studies to determine the optimal electroporation settings, depending on the type of machine and antigen used.
7. Ensure there are no air bubbles present by tapping on side of syringe with thumb and expelling air bubble by slowly depressing plunger until only DNA/saline solution is present in syringe.
8. Animals should be anesthetized prior to vaccination, as when awake, they may contract their muscles, thereby squeezing the DNA vaccine out. This also limits the possibility of animals struggling or biting during injection.
9. Good contact is required for the electroporation procedure to work properly. Ensure that electrodes are properly inserted and that a consistent current/resistance is being registered before delivering the pulse. It may be necessary to recruit a helper to work the electropulse generator depending on the model used. Some generators have an available foot pedal that allows a single operator to both align the electrode array and trigger the machine. However, many generators lack this accessory. In this case, it may be too cumbersome for a single person to hold the electrode array in place and work the controls.
10. The thickness of the skin can directly impact the level of plasmid gene expression. Ensure that vaccination sites are of consistent thickness by using a central location and keeping away from the belly of the animal.
11. Intradermal injection should result in a bleb in the skin that persists for several minutes. If this does not appear, or quickly dissipates, the injection has most likely been given subcutaneously. This will result in poor immune responses.
12. In addition to electroporation, there are several other DNA vaccine delivery methods that have been experimented with to varying degrees of success. Needle-free injection systems such as bio-injectors deliver vaccine plasmid by forcing a liquid stream through the skin, resulting in either intradermal or intramuscular delivery. These systems yield wider distribution of the vaccine, and increased antigen

production than do straight IM or ID injection [19, 20]. Delivery systems such as nanoparticles and liposomes have shown promise, but have certain drawbacks related to difficulty of formulation and delivery, particularly when it comes to vaccination in the field [21-30]. Another method that is gaining interest is the formulation of DNA launched virus-like particles (VLP). In this case, the antigen is encoded in one DNA plasmid, while structural proteins are encoded in a second plasmid. Transfection of a cell by both plasmids allows for expression of endogenously produced VLPs. This allows for increased extracellular bioavailability of the antigen and therefore increased immunogenicity [31].

Disclaimer

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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